NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION, AND REPAIR
Annual Technical Report
1994

Submitted by

Nicolas G. Bazan, M.D., Ph.D.
Project Director

Period Covered: 20 September, 1993, through 19 September, 1994

Cooperative Agreement DAMD17-93-V-3013

between

United States Army Research and Development Command
(Walter Reed Army Institute of Research)

and

Louisiana State University Medical Center
Neuroscience Center of Excellence

CORE RESEARCH FACILITY

Project Directors:
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Roger Beuerman, Ph.D.

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13. ABSTRACT *(Maximum 200 words)*

The LSU Neuroscience Center is a comprehensive, multidisciplinary, and transdepartmental entity that unites fundamental neurobiology and the clinical neurosciences in the common goal of elucidating the workings of the brain and contributing to the treatment of currently incurable diseases of the nervous system. The objective of the present program is to find solutions to neuroscience-related problems of interest to the U.S. Army Medical Research and Development Command. The program is focused on exploiting novel neuroprotective strategies that lead to prevention of and repair after neural injury. Converging approaches using state-of-the-art tools of cell biology, neurochemistry, neuroimmunology, neurophysiology, neuropharmacology, molecular biology and virology are proposed. Over the next four years, this program aims to: 1) carry out seven research projects in the basic and clinical neurosciences; 2) expand central, shared facilities with the addition of highly specialized instrumentation not currently available to our scientists; 3) develop laboratory space to permit the physical consolidation and coordination of this research effort; and 4) institute a coordination unit to monitor, facilitate, and administrate the cooperative research programs, as well as to meet the associated budgetary, human resources, facilities, and communications needs for the attainment of the proposed program goals.

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This Technical Report covers the progress made in the first year of this Cooperative Agreement in one project of the original proposal. We hope that this format of the report will facilitate its handling. The table of contents for all the projects has been included in each volume as well as letters from members of the External Advisory Committee of the LSU Neuroscience Center who have conducted an initial review of the work done supported by this Cooperative Agreement.

Nicolas G. Bazan, M.D., Ph.D.
Director, LSU Neuroscience Center
Program Director, USAMRDC Cooperative Agreement

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1. HSV-1 latently-infected mice display an altered response to stress: Implications for antiviral immunity.
2. Mouse lymphocytes express an orphan opioid receptor
3. Morphine suppresses peritoneal and splenic CTL activity in a dose-dependent fasion in alloimmunized mice
4. The frequency of exposure to morphine differentially affects CTL activity in alloimmunized mice.

Manuscripts:

1. Carr DJJ, Carpenter GW, Garza HH, Baker ML, Gebhart BM (in press) Cellular mechanisms involved in morphine-mediated suppression of CTL activity. In: *The Brain Immune Axis in Substance Abuse* (Sharp, Friedman, Maddin and Eisenstein, eds), Plenum Press.
2. Carpenter GW and Car DJJ (submitted) Pretreatment with β-funaltrexamine blocks morphine-mediated suppression of CTL activity in alloimmunized mice.
3. Carr DJJ and Carpenter GW (submitted) Morphine-induced suppression of spenic CTL activity in alloimmunized mice is not mediated through aδ-opioid receptor.
4. Carpenter GW, Garza HH, Gebhardt BM, Car DJJ (in press) Chronic morphine treatment suppresses CTL-mediated cytolysis, granulation and cAMP responses to alloantigen

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Project Director: Nicolas G. Bazan, M.D., Ph.D.

Participating Scientists: Geoffrey Allen, Ph.D.
Gary D. Clark, M.D.
Victor Marcheselli, M.S.
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Participating Scientists: Claude A. Burgoyne, M.D.
Emily Varnell
Mandi Conway, M.D.

Manuscripts

1. Chew SJ, Beuerman RW, Kaufman HE (in press) Real-time confocal microscopy of keratocyte activity in wound-healing after cryoablation in rabbit corneas. *Scanning* 16.

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Project Directors: Prescott Deininger, Ph.D.
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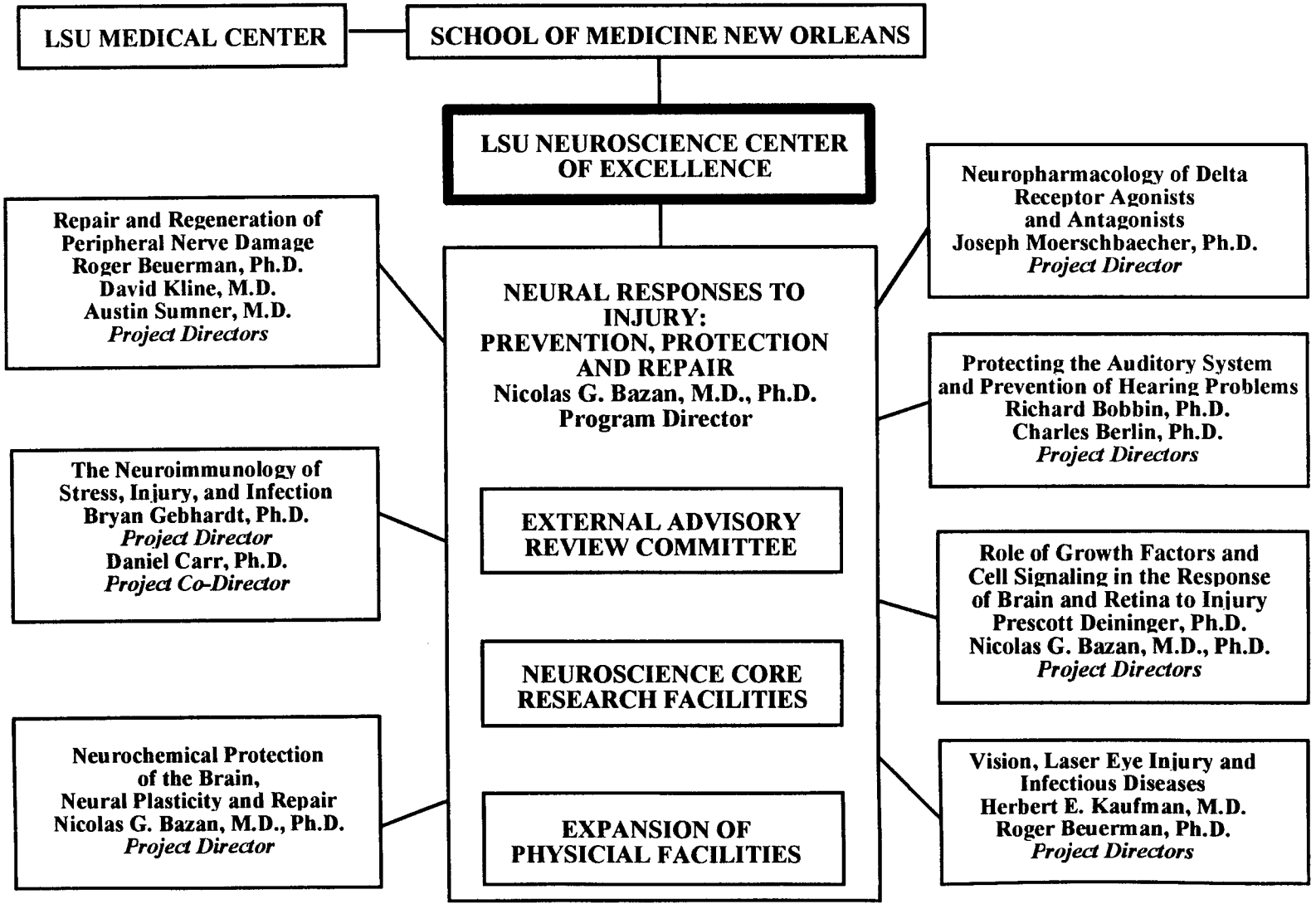
Cooperative Agreement Between the US Army Medical Research and Development Command and The LSU Neuroscience Center of Excellence

DAMD17-93-V-3013 20 September, 1993 - 19 October, 1997 $13,860,000



SCHOOL OF
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Louisiana State University
Medical Center
2020 Gravier Street, Suite "B"
New Orleans, LA 70112-2234
Telephone: (504) 568-6700
Telefax: (504) 568-5801

Neuroscience Center
Office of the Director

19 October, 1994

Commander
U.S. Army Medical Research and Development Command (USAMRDC)
ATTN: SGRD-RMI-S
Fort Detrick
Frederick, MD 21702-5012

Re: Annual report, Cooperative Agreement No. DAMD17-93-V-3013
Neural Responses to Injury: Prevention, Protection, and Repair

Dear Sir,

Please find enclosed the original and five copies of the first annual report for the Cooperative Agreement, referenced above, between the USAMRDC and the Louisiana State University Medical Center School of Medicine, Neuroscience Center of Excellence. This report represents the research carried out during the first year of this agreement (20 September, 1993, to date). It is organized per project, each corresponding to a chapter of the original application.

In addition to the research conducted in the first year of this agreement, the planning for the two additional floors of research space which are to be added to the Lions/LSU Clinics Building, 2020 Gravier Street, New Orleans, LA, has been completed, including all specifications necessary for the start of bidding. Enclosed is one copy each of the program manual (1 vol.) and the project manual (3 vols.) which has been generated by Cimini, Meric and Duplantier, Architects and Planners, for bidding purposes. It should be noted that there will actually be three floors constructed in this one project, two as funded by this Cooperative Agreement and one which is funded by LSU to be used by the School of Medicine for other purposes.

As planned, I arranged to have three meetings between the LSU investigators and their counterparts in the Army to provide program briefings for the work that they were planning to conduct under this agreement as well as to exchange ideas and information of mutual interest. The agendas for each of these meetings are enclosed. These provided both the LSU scientists and those of the Army the opportunity to discuss the work being done, the direction, and the significance to problems of interest to the Department of Defense.

On 2 December, 1993, several of our investigators, excluding the Auditory and Laser/Vision groups, met at the Walter Reed Army Institute of Research, Washington, D.C., with Drs. Frank Tortella, Joseph Long, Mark DeCoster and Jit Dave. These discussions revolved around the neurochemical and neuropharmacological aspects of the program project and provided a forum for the Army scientists tobegin interactions and exchange of information with our investigators.

On 31 January, 1994, the LSU auditory physiology group, represented by Drs. Charles Berlin and Richard Bobbin, and I met at Fort Rucker, AL, with Dr. Kent Kimball and Dr. Ben T. Mozo. These meetings involved presentations and discussions about the protection of the auditory system and prevention of hearing problems in humans.

The LSU investigators involved with the vision research, composed of Dr. Herbert Kaufman, Dr. Roger Beuerman and myself, met on 7 February, 1994, at Brooks Air Force Base, San Antonio, TX. These scientists and those of the Ocular Hazards Research Unit of the US Army Medical Research Detachment made presentations and conducted discussions focused on protection from, repair of, and prevention of laser injuries, specifically to the eye. Each of these information exchanges provided very useful direction and advice for the LSU investigators. These workshops will be conducted annually for the term of this agreement.

At the end of the first year of this program, as planned, I requested that two of the members of the External Advisory Committee of the LSU Neuroscience Center, Dr. Dennis W. Choi, Jones Professor and Head of the Department of Neurology, Washington University School of Medicine, and Dr. Fred Plum, Anne Parrish Titzell Professor and Chairman of the Department of Neurology, Cornell University Medical College, provide a critical review and a written report of the progress of the research accomplished under this Cooperative Agreement. Dr. Choi was given a copy of this annual report and subsequently made a site visit on 15 September, 1994, to the LSU Neuroscience Center. (The agenda for his meeting is attached.) At that time he met with a number of the investigators and administrators involved with whom he discussed many facets of the research being performed under this Agreement. His opinion of the work being done is attached.

Dr. Fred Plum made a site visit on 26 September, 1994, having also been provided previously with a copy of this annual report. He was also given the opportunity to examine the research and other progress made under this agreement and his written critique is also attached. Please note that, near the end of his letter (bottom of page two, first four paragraphs of page 5), Dr. Plum also included a description of projects not directly supported by the Cooperative Agreement but which are very positively impacted by any support of Neuroscience projects. The

reviewers were very complimentary of the positive consequences resulting from this support.

We are very pleased with the progress that has been made. We would like to thank you for the assistance you have given us. Please let me know if there is any further information that I can provide you.

Sincerely,

Nicolas G. Bazan, M.D., Ph.D.
Villere Professor of Ophthalmology,
Biochemistry and Molecular Biology,
and Neurology
Director, LSU Neuroscience Center

NGB/eht
enclosures

JOINT WORKSHOP ON "NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION AND REPAIR"

Sponsored by the LSU Neuroscience Center and Walter Reed Army Institute of Research, Department of Medical Neurosciences

December 2, 1993
Building 40, Room 2133

"Overview of LSU Program" **N. Bazan**	9:00
"Repair and Regeneration of Peripheral Nerve Damage" **R. Beuerman, D. Kline, J. England**	9:20
"The Neuroimmunology of Stress, Injury and Infection" **D. Carr**	10:10
Break	10:20
"Neurochemical Protection of the Brain, Neural Plasticity and Repair" **N. Bazan**	10:40
"Neuropharmacology of Delta Receptor Agonists and Antagonists" **J. Moerschbaecher**	11:15
"Stress and the Dopamine System" **J. Rao**	11:45
Box Lunch Served ($2.00 each)	12:00
"Role of Growth Factors and Cell Signaling in the Response of Brain and Retina to Injury **N. Bazan and J. Cook**	12:10
"An Overview of Neuropharmacology Research at WRAIR on Nervous System Injury and Protection" **Frank Tortella**	13:00
"Animal Models of Spinal Cord Injury and Mechanisms of Blood Flow Changes" **Joseph Long**	13:30
"Evaluation of Excitatory Amino Acids in Neuronhal Cell Culture" **CPT DeCoster**	13:50
"Molecular Biology of Nervous System" **Jit Dave**	14:10
Overall Discussion	14:30
Adjourn	15:00

zwp51\army\workshop.al

Joint Workshop on Neural Responses to Injury:
Prevention, Protection and Repair
Walter Reed Army Institute of Research, Dept. of Medical Neuroscience
U.S. Army Aeromedical Research Laboratory, Fort Rucker, AL
SCHEDULE FOR JANUARY 31, 1994

January 30

12:00 PM - depart New Orleans by car

Hotel: **Comfort Inn, 615 Boll Weevil Circle, Enterprise, AL 36330**
Tel. 205-393-2304, Fax. 205-347-5954

January 31

Visiting - Dr. Kent Kimball, Director, Plans and Programs, USAARL
Dr. Ben T. Mozo, Research Physicist, USAARL
Fort Rucker, AL 36362-5292
Tel. (205) 255-6917, Fax. (205) 255-6937

9:00 AM - Welcome
9:20 AM - Overview of LSU Program - **Nicolas G. Bazan**
9:45 AM - Protection the Auditory System and Prevention of Hearing Problem via Efferent Activation in Humans - **Charles Berlin**
10:30 AM - Break
11:00 AM - Prevention of Hearing Problems in Animals - **Richard Bobbin**
12:00 PM - General Discussion and Lunch
13:00 PM - Adjourn

OCULAR HAZARDS RESEARCH
U.S. ARMY MEDICAL RESEARCH DETACHMENT
7914 A DRIVE (Bldg 176)
BROOKS AIR FORCE BASE, TEXAS 78235-5138

February 7, 1994

Leave New Orleans on Continental flight #1445 at 6:00 PM, arrive San Antonio on Continental flight #1120 at 8:53 PM.

Hyatt Regency San Antonio
123 Losoya St., San Antonio, TX 78205
Confirmation #HY0000605552

February 8, 1994

8:30 *Overview of USAMRD program*
Bruce Stuck, Director, USAMRD

8:45 *Review of Accidental Laser Exposures and Human Tissue Response*
Donald Gagliano, Commander, USAMRD

9:00 *Overview of LSU Program*
Nicolas G. Bazan, Director, LSU Neuroscience Center

9:10 *The Program: Vision, Laser Eye Injury, and Infectious Diseases*
Herbert Kaufman, Chairman, Ophthalmology Dept. LSU

10:00 *Confocal Approach to Cellular Reactions in Wound Healing and of the Lamina Cibrosa.*
Roger Beuerman of the LSU Neuroscience Center

10:30 **BREAK AND LAB TOUR**

10:50 *Neurochemical Protection of the Brain, Neural Plasticity, and Repair*
Nicolas Bazan, Director, LSU Neuroscience Center

11:40 *Basic Fibroblast Growth Factor (bFGF) Treatment of Laser-Injured Retina*
Steven T. Schuschereba, Chief, Biology Section, USAMRD

12:10 *Role of Growth Factors and Cell Signaling in the Response of Brain and Retina to Injury: Focus on the Retina*
Nicolas Bazan, Director, LSU Neuroscience Center

12:50 **LUNCH**

2:50 Depart San Antonio on Southwest flight #803

5:55 Arrive New Orleans on Southwest flight #1055

LETTERS FROM MEMBERS OF THE EXTERNAL ADVISORY COMMITTEE

WASHINGTON UNIVERSITY SCHOOL OF MEDICINE
AT WASHINGTON UNIVERSITY MEDICAL CENTER

NEUROLOGY

Dennis W. Choi, M.D., Ph.D.

Andrew B. and Gretchen P. Jones Professor and Head
Neurologist-in-Chief, Barnes Hospital

October 17, 1994

Nicholas G. Bazan, MD, PhD
Director, LSU Neuroscience Center
School of Medicine in New Orleans
Louisiana State University Medical Center
2020 Gravier Street, Suite "B"
New Orleans, LA 70112-2234

Dear Nick:

Thank you for the invitation to visit LSU on September 15 and review early progress made under the LSU Neuroscience Center of Excellence Cooperative Agreement with the U.S. Army Medical Research and Development Command.

You have assembled an impressive array of faculty researchers to study diverse aspects of nervous system injury. Overall, I find the individual projects to be thoughtful and well chosen. With you as director, I am sure that they will be most ably integrated. Your project 3 "Neurochemical Protection of the Brain, Neuroplasticity and Repair" is in my view the clear focal point of the overall program. The identification of new PAF antagonist drugs capable of regulating excitatory synaptic transmission and excitotoxic central nervous system injury, is an attractive and attainable goal. The novel pharmacology theme is also well developed in Dr. Moerschbaecher's Section 4 "Neuropharmacology of Delta Receptor Agonist and Antagonist". Involvement of clinician-investigators in clinical departments, such as Dr. Sumner in Project 1 or Dr. Kaufman in Project 5 are strengths of the program that will enhance its ability to identify human therapeutic interventions.

Progress in the first months of operation appears to be on target. Substantial synergy can be expected between the research programs specifically outlined in this collaborative agreement, and the larger intellectual framework formed the LSU Neuroscience Center of Excellence. Your role as director of both efforts is a vital feature that will ensure maximization of this synergy. In summary, I am most enthusiastic about this LSU-U.S. Army Cooperative Agreement, both for its specific merit and as a prototype mechanism for facilitating effective collaboration between academic and military institutions.

Best regards.

Sincerely,

Dennis Choi

Box 8111
660 South Euclid Avenue
St. Louis, Missouri 63110
(314) 362-7175 • FAX (314) 362-2826

525 EAST 68th STREET NEW YORK N.Y. 10021

THE NEW YORK HOSPITAL-CORNELL MEDICAL CENTER

FRED PLUM, M.D., CHAIRMAN
ANNE PARRISH TITZELL PROFESSOR OF NEUROLOGY
CORNELL UNIVERSITY MEDICAL COLLEGE
NEUROLOGIST-IN-CHIEF
THE NEW YORK HOSPITAL- CORNELL MEDICAL CENTER
(212) 746-6141
FAX (212) 746-8532

September 28, 1994

Nicholas G. Bazan, M.D., Ph.D.
LSU Neuroscience Center
2020 Gravier Street
Suite B
New Orleans, LA 70112-2234

Dear Dr. Bazan:

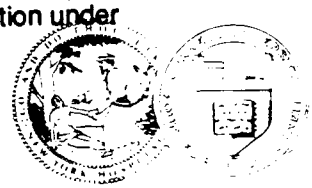
I am pleased to submit this reviewer's report of a Cooperative Agreement between the LSU Neuroscience Center and the US Department of the Army entitled, "Neural Response to Injury: Prevention, Protection and Repair" (henceforth designated as "Injury Study"). The agreement will span four years of effort by the LSU Center; this report describes progress obtained during its first year, extending from September 1, 1993 to August 31, 1994.

Nicholas G. Bazan, M.D., Ph.D. both directs the LSU Neuroscience Center of Excellence and serves as the Program Director of the Injury Study. In addition to Dr. Bazan's personal investigative efforts, seven additional study groups are engaged in research directly related to the Injury Study, as indicated in the administrative diagram attached to this report.

Dr. Bazan's outstanding personal and scientific qualities are the two most important factors in assuring the future success of the LSU-U.S. Army Cooperative Agreement. His leadership and intellectual "taste", as well as his joy in and dedication to brain science penetrate every aspect of the LSU Neuroscience Institute. His enthusiasm has spread to infect his colleagues and many other departments of the Medical School with his high scientific standards and integrity. His knowledge suffuses every dimension of basic neuroscience. His diplomacy and gentle handling of his staff creates their huge loyalty. His energy is contagious. Furthermore, he has the wonderful quality of scientific generosity: always ready to help and encourage others, he is entirely responsible for the continuously improving quality of young persons who are coming to LSU to learn and do important neuroscience.

In addition to the above, Dr. Bazan's specific research is internationally recognized as being of the highest caliber. His personal research contributions to the Injury Study during the past year reflects these high qualities in several ways. They have been published in the most competitively prestigious biomedical research journals. They also add new understandings to both the normal and potentially abnormal effects of the platelet-activating factor (PAF). PAF already is known to be a potent mediator of inflammatory and immune responses. What Bazan and his team now have found is that in low concentrations, PAF transmission may enhance memory and repair mechanisms in brain. Alternately, if released in excessively large concentrations or in combination with certain other molecules, PAF appears capable of causing immune-related tissue damage such as occurs with intense inflammation and/or the induction of genetic prostaglandin synthesis, a step that also may injure brain tissue. This fundamental research emphasizes the complexity and often bidirectional responses that may occur when injury strikes the brain. The results are important and illustrate the difficulties which must be overcome in establishing prevention, protection and repair of brain injuries.

Drs. Bazan and Prescott Deininger have succeeded in developing a series of transgenic mice expressing a dominant mutant of platelet derived growth factor (PDGF). Remarkably enough, the animals thus far have shown no major behavioral alteration under



normal developmental conditions. Their reaction to ischemia, seizures and other circumstances has not yet been tested.

Let me turn now to some of the other, supporting projects: **Drs. R. Benerman, D. Kline and A. Sumner** have made good progress in their studies of neurotrophic factors and other mechanisms in human and experimental neuromas resulting from blunt and crush nerve injuries. Basic fibroblast growth factor (bFGF) was the most prominent factor found in human post-nerve injury neuromas with other specific factors either absent or reaching only very low levels of concentration. More precisely analytic experiments await the analyses of fresh neuronal material from the experimental preparations.

Drs. Herbert Kaufman and Roger Benerman have made brilliant advances using confocal microscopy to examine the cellular details of the human retina. To a degree never before possible they have safely demonstrated in awake human subjects the acute pathophysiology of laser injuries to cornea and their early transformation into fibroblasts. Detailed identification of anterior chamber cells has been possible and current efforts are underway to examine at great magnification the optic disc itself. Ocular fungus and herpes infections can be identified immediately and without introducing foreign substances against the cornea or into the eye. Application of the tool should have an important place in clinically applied military medicine.

During the past year, the investigators also have pursued their earlier discovery that ambient chilling of monkeys latently infected with H. Simplex induces an acute recurrence of cutaneous herpes. Furthermore, chronic ingestion of the beta blocker, propanelol, has been found to ameliorate or prevent the active recurrence. Clinical trials of this important discovery must be pursued as it has important practical aspects.

During the year, the necessary work to establish and equip the glaucoma research laboratory was undertaken. Next year's report can be expected to provide research results from that laboratory.

Dr. Joseph Moerschbaecher and his colleagues in pharmacology have initiated preliminary studies on the influence of delta opoid agonists-antagonists on learning and antinociception. Somewhat surprisingly, the agent damps the CO_2 response of breathing but has no antinociceptive effect. The same investigator is analyzing how anxiogenic drugs affect dopamine neurons in the ventral tegmental area of the rodent brain.

In another preliminary approach, **Drs. H.W. Thompson et al** have initiated experiments passing retroviral gene carriers into the eye with externally applied eye drops, thereby developing a new approach to deliver protection against certain ophthalmologic infections or enhancing the potential success of corneal transplant.

Drs. Richard Bobbin and Charles Berlin, thanks to the DOD grant, have added an excellent postdoctoral student as well as important new equipment to their laboratory. The laboratory's principal subject of interest is to find mechanisms for preventing the audiologic damage produced by intense sound. In guinea pigs, this has been achieved by stimulating calcium-dependent mechanisms in cochlear neurons. In another study, the laboratory has found in human studies that during the delivery of loud, binaural sounds, men and women suppress the noise in opposite sided ears from each other.

The above individual achievements provide only a part of the considerable effort, enthusiasm and success that the U.S. Army grant has brought to the LSU Neuroscience Center of Excellence (NCE). The following steps forward can also be emphasized:

1) Morale in the LSU-NCE rides at high pitch, encouraging scientific collaboration and the generation of new ideas.

2) Funds have been granted to subsidize the necessary equipment and technical personnel to establish a brain bank. Presently, approximately 50 specimens are available in storage with the Center holding good clinical records of the preterminal illness.

3) A program of "starter" grants designed to assist young investigators in conducting merit-deserving, self designed research projects has been initiated.

4) A highly popular state-wide Graduate School outreach summer program has been successfully concluded, attracting a strong interest in neuroscience among gifted college students.

5) An interdisciplinary graduate program in neuroscience was initiated and strongly encouraged by the faculty during 1993-94. As a result, nearly all of the graduate students (including the new entering class) are of very good quality. Indeed, other participating departments say that the Neuroscience graduate students are the best among the LSU biological sciences programs.

Summary. Under the generous auspices of a U.S. Army Cooperative Agreement, the LSU Neuroscience Center of Excellence is not only thriving but headed for far greater future productivity than at any time in the past. The admirable success of the program depends heavily on the foresight, intelligence, creativity and energy of two outstanding scientists, Herbert Kaufman and, especially, Nicholas G. Bazan. Their achievements and those of their colleagues totally warrant continuation of support. Indeed, every indication is that their extramural, non-Army support will continue to grow, making the program stronger and stronger as the years elapse.

One serious problem remains - that of sufficient space in which to do the studies that Dr. Bazan and his colleagues already have conceived so well. Prompt attention to and effective application of must be given to the DOD funds already awarded to construct new research space which will greatly increase the LSU Neuroscience team's opportunities for creative discovery.

I and my colleagues on the External Advisory Board of the LSU Neuroscience Center of Excellence strongly endorse the quality and number of achievements that have come from the U.S. Army-LSU-NCE collaboration. Thanks to strong leadership for the Center and a high degree of internally high morale and interdependence within the Center, it can be anticipated that the Cooperative Agreement will have a major impact on national neuroscience research as well as the specific medical needs of the U.S. Army.

Sincerely,

Fred Plum

Fred Plum, M.D.

FP/moc

CONFOCAL MICROSCOPY AND CELLULAR IMAGING

I. Personnel:

As a first step in establishing a core facility for real time cellular imaging and confocal microscopy, a faculty member was appointed who would use the facility to accomplish research goals of the Neuroscience Center, as well as to manage collaborative projects within and outside of the university. The interview process included a scientific seminar and discussions with several faculty at the LSU Medical Center. Dr. Mark A. DeCoster was hired for this faculty position as a Research Assistant Professor. Dr. DeCoster is an expert in calcium imaging and has substantial experience in managing core facilities for imaging. He received his Ph. D. in Biochemistry and Molecular Biophysics from the Medical College of Virginia, Richmond, VA in 1989. He was a Research Biochemist at the Walter Reed Army Institute of Research, Department of Medical Neurosciences, Washington, DC from 1989-1994. At Walter Reed, Dr. DeCoster was one of two scientists trained in the use of the Institute's interactive laser cytometer, which he used for calcium imaging. He later became the first non-commercial user of Meridian Instruments, Inc.'s laser scanning confocal microscope, which he set up in an imaging laboratory at Walter Reed. He used this system for confocal and real-time imaging experiments of excitatory amino acid-induced calcium changes in primary neuronal cultures, as well as for measurement of spontaneous neuronal calcium oscillations. Dr. DeCoster also established collaborations using this system with scientists from NIH and the FDA. Dr. DeCoster has published 11 papers, dealing with calcium flux in primary neuronal cultures, neurotoxic and neuroprotective actions and mechanisms, second

messengers and neuronal development, and Schwann cell mitogenic factors.

II. Facilities: Real Time Cellular Imaging and Confocal Microscopy:

The NORAN system has been chosen for accomplishing real time cellular imaging and confocal microscopy of neuronal cultures and tissues at the Neuroscience Center. Primary goals will include evaluating calcium and hydrogen ion (pH) changes in nervous tissue in the normal, and injured state. The NORAN system is ideally suited for this task by virtue of its visible and ultraviolet laser modes (allowing for ratiometric calcium measurements), scanning speed, and Silicon Graphics compatibility. The importance of Silicon Graphics compatibility is magnified by the developing Silicon Graphics workstation network being set up throughout Louisiana for biological imaging applications.

Approximately 220 sq. ft. of space within the Neuroscience Center has been allocated for the Core Imaging Facility. In addition to the digital video confocal laser scanning imaging system purchased from NORAN, several other pieces of equipment have been purchased for the Imaging Facility. These include, a Nikon Diaphot inverted microscope, equipped with phase optics and epifluorescence, a TMC airtable for vibration isolation, and a Focus Graphics Imagecorder film recorder and Imageprinter dye sublimation printer for hardcopy output. In addition, software and computer support has been purchased to allow data analysis and modeling. All equipment mentioned has been received, and system installation was begun 22 August 1994 and completed 26 August 1994.

Preliminary experiments with the Noran system have demonstrated the speed and

sensitivity of the system in recording calcium changes in living neurons. Basal, unstimulated calcium oscillations have been observed, as well as calcium flux caused by exogenous stimuli such as KCl, glutamate, or PAF addition. The data obtained so far using the Noran system have been very exciting, and are truly state-of-the art. However, while the results from our experiments have been positive, the speed and resolution of the Noran system were not fully appreciated until we started using the system. While Noran will continue to make improvements in the software supporting their confocal microscope system, we are certain that a number of hardware and software upgrades need to be added to the system now, to keep this project moving. Three major upgrades are required, as outlined below:

1. External hard disk, 2 Gigabytes: The Silicon Graphics Indy workstation running the Noran system was purchased with a 1 Gigabyte internal hard disk. Since the Silicon Graphics computers switch memory between RAM and the hard disk, keeping some of the hard disk space free for this purpose is essential. Approximately 60 % of the internal hard disk is taken up by essential software running the system; this leave 400 Megabytes (Mb) for data collection and storage. A typical 150 second recording of living neurons at a scan rate of 2 secs/ frame collects 75 frames. At a resolution of 640 x 480 pixels, these 75 frames require 22 Mb of storage space. This single 22 Mb file would allow the recording of basal calcium oscillations, for example, followed by addition of an experimental compound and a positive control in one well of neurons, with analysis of 10-30 neurons allowed in the digitized field of analysis. This experimental well would need to be followed by a second well to which a negative control was added, to validate the specificity of the first result. This negative control well would be carried out under the same conditions as the positive control, and would thus require an

additional 22 Mb of storage space. To duplicate this result on the same day would require another pair of wells, or 44 Mb more of storage space. Thus, to duplicate this one concentration effect with appropriate negative controls would require 88 Mb of storage space, or 22% of that available on the entire hard disk. We are now typically carrying out 3-4 sets of conditions or concentrations per day; this requires 66-88% of the free hard disk space. To analyze or even review the data efficiently, the experiments must be moved off one at a time to a 128 Mb rewritable optical disk drive which we have connected to the system. Each optical disk must be formatted to the specific system and mounted; this process takes about 15 minutes. Moving each file takes about 5 minutes, and typically, only 5 files fit on a disk. Therefore, moving a typical day's data off the hard disk so that the system will run more efficiently, in itself takes a couple of hours. We thus propose the addition of a 2 Gigabyte external hard disk to the Noran system. This would allow the fast and efficient transfer of collected data off of the internal hard disk, thus allowing the system to run more efficiently for more data collection that day or for analysis of collected data. Data that had been analyzed could then be archived to the 128 Mb optical disks.

2. Third photomultiplier tube (PMT): The current configuration of the Noran system at the Neuroscience Center includes two PMTs. This configuration allows us two record the ratio of one dye (allowing for ratiometric quantification of ion concentration), or the relative intensities of two dyes (qualitative data). However, we feel it is essential to investigate not only calcium or pH changes in the cell during neurotrauma, but rather, to investigate simultaneously calcium and pH or calcium and sodium. A third PMT would allow these, and other combinations of simultaneous multi-indicator measurements to be carried out.

3. Second license agreement with Noran: The Neuroscience Center is part of an intrastate networking project which will allow internet linkage of Silicon Graphics workstations here at LSU in New Orleans, as well as at other locations in Louisiana. As part of this project, the Neuroscience Center will be receiving a second Indy workstation similar to the one running the Noran system. We propose to use this second Indy as a workstation for analysis of data collected on the Noran system. Since data analysis is labor intensive, it currently ties up the Noran system, both in computer memory and computer time, inhibiting the use of the Noran system for data collection. Collected data could be moved off of the Noran system onto the second Indy workstation via the internet, freeing up computer memory and computer time on the Noran system.

III. Equipment for Electrophysiological Studies

Approximately 35% of the laboratory space in the Neuroscience Center imaging facility is also equipped with state-of-the-art electrophysiology equipment for patch clamping and voltage recordings from neuronal tissues. This equipment will allow for correlative experiments comparing voltage recordings and ionic imaging of neuronal tissues in the normal and injured states.

IV. Proposed Projects

Drs. DeCoster and Bazan plan to use primary neuronal cultures from rat embryos to study the effects of platelet-activating factor (PAF) on calcium ($[Ca^{2+}]_i$) and hydrogen ion (pH) concentrations in neurons. Dr. DeCoster has successfully established these primary cultures at the Neuroscience Center. The previously characterized effects of excitatory amino acids (EAAs) on the cellular dynamics of these ion concentrations in neurons will be utilized as positive controls; namely, EAAs such as glutamate and N-methyl-D-aspartate (NMDA) will be used at first to characterize neuronal ionic and viability responses. Then, in identical sister cultures, PAF will be added to neurons, and ionic and viability responses measured.

With the development of fluorescent PAF analogs, it is proposed that confocal observation of cellular activation and transport of this lipid messenger will be possible using the NORAN system. Both normal incorporation of these PAF probes as well as that after neuronal injury will be carried out. Another utility of the NORAN system combined with developed fluorescent probes is the intracellular release of caged compounds. These compounds are released by ultraviolet photolysis via the NORAN system laser. Caged arachidonic acid probes are already commercially available; caged PAF is available through collaboration and caged PAF antagonists may be developed in the future. All of these compounds would provide new levels of temporal resolution to address PAF-mediated cellular signalling pathways. In addition, caged calcium chelators will be used to address the role of local $[Ca^{2+}]_i$ changes in the neuron.

The other major system to test with the imaging facility resources will be the brain slice preparation, for example, from hippocampus. After working out the technical aspects of imaging, similar experiments to those outlined above for cell culture will be carried out on slice preparations. In addition, the synaptic integrity of the slice preparation will allow

voltage-sensitive dyes to be used to address circuit function and failure under normal and traumatic conditions, respectively. Other planned areas of research utilizing the slice preparation would address the cellular changes occurring after kindling response, and genetic alterations, for example, as measured via the expression of immediate early genes (IEGs).

The confocal microscopy and cellular imaging core facility at the Neuroscience Center will provide major support to the projects: "Role of Growth Factors and Cell Signaling in the Response of Brain and Retina to Injury", and "Neurochemical Protection of the Brain, Neural Plasticity and Repair".

Below is a summary of initial experiments underway utilizing the primary rat neuronal cultures and the Noran imaging system.

Experimental groups:

1. Platelet activating factor (PAF)
2. Phospholipases A_2 (PLA_2)
   a. OS_1
   b. OS_2
   c. bee venom
3. PAF + PAF-antagonists
4. PAF + calcium channel antagonists
5. PAF + glutamate
6. PLA_2s + PAF-antagonists
7. PLA_2s + calcium channel antagonists

8. glutamate + PAF antagonists

9. PAF + glutamate antagonists

10. (PAF + glutamate) + PAF antagonists

11. (PAF + glutamate) + glutamate antagonists

Control groups:

1. lyso-PAF

2. lyso-PAF + PAF antagonists

3. glutamate

4. glutamate + glutamate antagonists

Justification:

1. The primary lipid messenger to be studied is PAF. Major areas of investigation involving PAF will be measurement of intracellular calcium concentration ($[Ca^{2+}]_i$) in neuronal cultures before and after PAF addition, cellular hydrogen ion concentration (pH) changes under the same conditions, simultaneous $[Ca^{2+}]_i$ and pH measurements, and measurement of cell viability/death after PAF addition.

2. a-c. PLA_2 receptors have recently been isolated from both muscle and brain tissue. These receptors are thought to bind secreted forms of PLA_2 which may then activate the release of lipid messengers. OS_1, OS_2, and bee venom are distinct agonists at these PLA_2 receptors, and will be used to determine if they release lipid messengers in our culture system. One of these

agonists, OS_2, has been shown to bind to brain tissue.

3. Once $[Ca^{2+}]_i$/pH and cell viability changes in response to PAF alone have been determined (group 1), PAF-antagonists available to us will be used to determine the specificity of these changes to PAF receptors.

4. Similar to group 3, we have available to us novel calcium channel antagonists, which we will use to attempt to block PAF cellular signalling.

5. Glutamate is a positive control for $[Ca^{2+}]_i$ and cell viability changes in neuronal cultures (see control group 3 below). Dr. DeCoster has published numerous scientific articles on these effects of glutamate. We will therefore determine if co-treatment with PAF and glutamate leads to different cellular changes than with PAF treatment alone. Also, this will help isolate whether PAF effects are leading to endogenous glutamate effects, which Dr. DeCoster has shown occurs when neuronal cultures are treated with other stimuli, such as KCl depolarization. On the other hand, this paradigm may also help determine if glutamate effects interfere with or modulate those caused by PAF alone.

6. If PLA_2s are shown to cause release of lipid messengers in our culture system (see 2a-c), then PAF antagonists, characterized under group 3, will be used to study possible antagonism of PLA_2 effects.

7. Once calcium channel antagonists have been characterized against PAF (group 4), they will be tested against PLA_2 effects.

8. Glutamate, a positive control for $[Ca^{2+}]_i$ and cell viability changes in neuronal cultures (see control group 3 below), is found in high concentrations of the mammalian CNS, and is the likely major excitatory amino acid neurotransmitter in humans. Since it has been previously shown by Dr. DeCoster that the neuronal cultures used in these studies contain

endogenous glutamate, release of this neurotransmitter may be modulating other responses measured in our proposed studies. Therefore, it will be essential to determine whether neuronal responses stimulated by addition of exogenous glutamate are blocked by PAF antagonists.

9. Since PAF addition to neuronal cultures may cause the release of endogenous glutamate, it must be determined whether PAF responses are blocked by glutamate antagonists.

10. Submaximal concentrations of PAF and glutamate co-treatment (group 5) may result in neuronal responses distinct from the responses to either agonist added alone. The results from group 5 will be used in group 10 to test whether unique co-treatment responses are blocked by PAF antagonists.

11. Similarly to group 10, it will be determined in group 11 whether unique co-treatment responses are blocked by glutamate antagonists.

Control groups:

1. Lyso-PAF is a metabolized form of PAF which has been shown to be inactive in comparison with PAF in many model systems. Since the affinity of lyso-PAF for the PAF receptor is low compared to PAF, and the molecular structure is very similar, this an important negative control compound for testing PAF receptor specificity.

2. A possible cellular responses to lyso-PAF will be compared in the presence of PAF antagonists, to determine if they are non-specific, or mediated through a non-receptor mechanism.

3. Glutamate will be the positive control for $[Ca^{2+}]_i$, pH, and cell viability changes in this neuronal culture model. Dr. DeCoster has published numerous scientific articles

characterizing glutamate responses in these cultures.

4. The specificity of glutamate responses (control group 3) will be tested using glutamate antagonists.

CELL BIOLOGY

In the Cell Biology Section, three microscopes and a carbon evaporator have been purchased and set up. A **Nikon Optiphot-2** upright, compound microscope is being used to image histological sections of retina. Following our light damage protocol, retinal sections are treated for TUNEL, and apoptotic nuclei within the photoreceptor nuclear layer are localized. This process utilizes the Optiphot-2, and the Nikon imaging attachments: the Sony DXC-960MD CCD three-color camera, the Sony CMA-D2 camera adaptor, and the Sony Trinitron digital color monitor. TUNEL-stained DNA can be detected through the development of a color response, giving a pink-purple color to apoptotic nuclei. In addition, a peroxidase reaction allows the labeled DNA to appear dark brown, enabling the tissue to be analyzed at the electron microscope level following light analysis. Finally, use of a fluorescent tag produces high contrast fluorescent microscopy images that often facilitate analysis. The differential interference contrast (Nomarski) optics, the epi-fluorescent system with neoflur objectives, and the internal filtering of the Optiphot-2 has allowed us to acquire the best optical information possible. Images are direct-viewed in video form, where general counting occurs, but can also be digitized and placed on disc for later, additional analysis. This system

is being used extensively, resulting in the data presented in the light damage section.

In addition, histological sections of hippocampus are now being analyzed with this system. Following four vessel occlusion and ischemia-reperfusion in rodent, hippocampal slices are prepared. These are being compared with sham operated animals (controls) and animals treated with the PAF antagonist BN52021. Nuclei numbers and neural morphology, especially within the CA1 regions, are being analyzed to determine the initial degree of damage (and subsequent cell loss) and the neuroprotective effects of the PAF inhibitor. Image analysis, in this case, involves high resolution 80 μm-thick plastic sections of Golgi-treated silver-stained tissue, conventional 1 μm-thick toluidine blue-stained sections, and 10 μm-thick hematoxylin/eosin-stained paraffin sections. The imaging capabilities of this Nikon Optiphot-2 system allow us to rapidly obtain information on the changes occurring at the histological level.

A **Nikon Diaphot-200** inverted microscope, containing a complete set of high resolution, large numerical aperture objectives, has been coupled to our Noran Odyssey confocal microscope, completing this piece of equipment. A separate section discusses the confocal system.

The **Nikon SMZ-U** is used routinely for preparation of retinal and hippocampal samples. Using this microscope, tissue is fine-dissected and oriented, following preliminary fixation. This is especially useful in the case of hippocampal studies, since it is extremely important that the hippocampal CA1 cells are radially oriented prior to embedding and

sectioning. Finally, because of the magnification range of this microscope and the size of the hippocampal slices, we have found it extremely useful to use this microscope with the imaging system discussed above to provide access to the relatively large area of the hippocampal cross (radial) sections.

The high vacuum **carbon evaporator** has also been obtained and installed. This water cooled system now enables us to accurately place a 5 nm coating of carbon on electron microscope sections, prior to coating with high resolution electron microscope autoradiographic emulsion. Since these 800 Å-thick sections require several months to expose the emulsion to the energy of tritium disintegration, no preparations have yet been examined. However, the ease with which this equipment functions, and its proximity, insures a greatly shortened preparation period.